Research Article

Designing a Novel Chimeric Antigen to Stimulate the Immune System against *Brucella* Infection

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Abstract

Brucellosis is a well-known infectious disease among domestic animals caused by Brucella bacterium. OMP25 is an outer membrane protein of Brucella which plays an important role in immunogenicity of cells. Moreover, Brucella Lumazine Synthase (BLS) as another main antigen can be used as an adjuvant when covalently attached to a foreign antigen. In the current study, designing and production of a chimeric construct as a primary step to stimulate the immune system against Brucella infection is investigated. The construct was amplified by specific primers using SOE-PCR technique. The amplified construct was cloned in pTZ57R/T vector and transformed into E. coli TOP10F' as cloning host. Then, the construct was subcloned in pET-32a (+) vector followed by transforming into *E. coli* BL21 (DE3) as expression host. Our results demonstrated OMP25-BLS with 1163 bp was successfully cloned and expressed in the expression host. Results were confirmed using sequencing, SDS-PAGE and western blotting which correctly showed 59 KD protein band of OMP25-BLS. According to our results this construct could be proposed to investigate as a new subunit vaccine candidate in order to stimulate immune system against Brucellosis in future studies.

Key words: *Brucella melitensis* Rev1; Subunit vaccine; OMP25-BLS

Introduction

Brucellosis, a common zoonotic disease, is caused by *Brucella*, a gram negative coccobacillus that lacks capsule or flagellea as well as facultative intracellular (1). In animals, it is characterized by abortion and reduced fertility, while it has symptoms such as undulant fever, arthritis and osteomyelitis in humans (2). The genus of *Brucella* consists of more than ten species, although, *B*. *abortus*, *B*. *melitensis*, and *B*. *suis* cause most of the animal and human diseases. *B*. *melitensis* which mainly infects goats and sheep is considered as the most pathogenic species of *Brucella* to humans (3).

In animals, immunization against *Brucella* infection is usually performed by administration of the live attenuated smooth *Brucella* strains like *B*. *abortus* S19, *B*. *melitensis* Rev.1 and non-smooth strain *B*. *abortus* RB51 (4). There, however, exists some limitations regarding the attenuated vaccines that include abortion in immunized pregnant animals, being pathogenic for humans, and interfering with the lipopolysaccharides-based serological tests (5). Till now, there is no commercially available vaccine against human brucellosis and the disease is commonly prevented by immunization of uninfected animals

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and elimination of the infected ones (6). *Brucella* outer membrane proteins (OMPs) are specific cell surface antigens having remarkable immunogenicity characteristics (7). These cell surface antigens are classified into two main groups consisting of 1) OMP2a and OMP2b and 2) OMP25 and OMP31 (7). OMP25 antigen, as one of the virulent factors, is the major antigen involved in survival of *Brucella* and found to be highly conserved among different *Brucella* species (8,9). *Brucella* species that lack OMP25 have shown to be attenuated in cattle (10).

In addition, it was shown that OMP25 inhibits the production of TNF-α, which is an important component of the cytotoxic immune response in human macrophages infected by *B. suis* (11). BLS, as another *Brucella* antigen with important immunogenic characteristics, is also highly conserved among different species (5,9).

BLS has also been found effective as an adjuvant while linking to a foreign antigen covalently (5). Furthermore, BLS can activate the cellular responses by delivering CD8+ T-cell epitopes into the MHC class I pathway, and eliciting an antigen-specific response by production of IFN-ϒ (12). The aim of the present study was to design, clone and the expression analysis of chimeric OMP25-BLS antigen We, therefore, herewith, report a new chimeric construct as a primary step toward introducing new subunit vaccines in order to stimulate the immune system against *Brucella* infection.

Materials and Methods

Bacterial Strains, Growth Conditions and Isolation

In the current study, *B. melitensis* Rev 1 strain was obtained from the *B. melitensis* culture collection of Razi Institute (Mashhad, Iran) and cultured, as previously described (13). DNA was extracted using a DNA extraction kit (Bioneer Korea). The quality and purity of the extracted DNA were analyzed by agarose gel electrophoresis and NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA).

Construction of the Chimeric OMP25-BLS

The full length of OMP25 (642 bp) and BLS (477 bp) genes were already amplified and cloned in our previous studies (Figure 2A) (9,14), and used as template for amplifying chimeric OMP25-BLS construct using EX Taq DNA polymerase (Takara, Japan). In order to design the construct, specific primers with 30 bp complement linker (15) at the 5' end of reverse primer of OMP25 and the forward primer of BLS (as indicated in Table 1 by bold letters) and the restriction sites at the 5′ end (as underlined in Table 1) of the forward primer of OMP25 and the reverse primer of BLS were considered using Primer Premier 5 software.

Table 1: The sequence of specific primers with restriction sites

Gene	Primer sequences	Restriction enzyme
OMP25	`F:5`- *CCATGGCT*ATGCGCACTCTTC-3	*NcoI*
	`R:5`-***TTTAGCCGCTGCTTCTTTTGCCGCAGCTTC***GAACTTGTAGCCGATGC -3	
BLS	`F:5`-***GAAGCTGCGGCAAAAGAAGCAGCGGCTAAA***ATGAACCAAAGCTGTC-3	
	`R:5`- *GGATCC*TTATCAGACAAGCGCGGCGATGCG-3	*BamHI*

Polymerase chain reaction (PCR) was carried out by means of the Personal Cycler™ thermocycler (Biometra, Germany) and the spliced overlap expression by PCR (SOE-PCR) technique, as described in our previous study (Figure 1) (16). It contained 2.5µL of 10X PCR buffer, 2µL of $MgCl_2$ (50 mM), 2µL of dNTPs (2.5 pmol/µL), 0.3 µL of OMP25 or BLS construct in pTZ57R/T (50 to 100 ng/µL), 1.5µL of mix primer (5 pmol/µL), 0.125 U/µL of EX Taq DNA polymerase and deionised water up to 25µL reaction volume. The PCR program was performed with an initial denaturation at 94°C for 6 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec, extension at 72°C for 45 sec, and a final extension at 72°C for 10 min (Figure 1, step I).

The SOE-PCR step to amplify OMP25-BLS construct was carried out in 25 cycles of denaturation for 30 sec at 94˚C, annealing for 30 sec at 62˚C and extension for 45 sec at 72˚C. The initial denaturation and final extension were 5 min at 94˚C and 72˚C, respectively. The mixture of PCR contained 2.5µL of 10X PCR buffer, 2µL $MgCl_2$, 2µL of dNTPs, 0.5µL of the first PCR reaction mix, 1µL of mix primers (5 pmol/µL), 0.125 U/µL of EX Taq DNA polymerase and deionised water up to 25µL reaction volume (Figure 1, step II).

Cloning and Subcloning

The PCR products were purified from the agarose gel using Ron's Agarose Gel Mini Prep Kit protocol, according to the manufacturer's instructions (BioRon, Germany). And the purified products were ligated into pTZ57R/T cloning vector by T/A cloning strategy according to the manufacturer's instructions (Thermo Scientific, USA). *Escherichia coli* TOP10 F` competent cell preparation and transformation steps were followed as described by Sambrook and Russell (17).

The recombinant plasmids were transformed into competent *E. coli* TOP10 F'. The recombinant clone(s) and the harboring plasmid DNA with inserts were screened based on their ampicillin resistance. Ampicillin resistant colonies were grown in Luria-Bertani (LB) broth liquid medium containing 50 µg/ml of ampicillin at 37°C. The fidelity of *E. coli* TOP10 F' transformants were verified by colony-PCR using M13 universal primers. The recombinant plasmids were purified using the Ron`s Plasmid Mini Kit (BioRon, Germany), and confirmed by restriction enzyme digestion. Purified plasmids were then subjected to sequencing (Bioneer, South Korea).

In order to subclone genes into expression vector, pET-32a(+), purified digested products were cloned into the expression vector in which the recombinant protein included a six-Histidine tag (His-tag) at the N-terminal for easier purification. Standard techniques for these steps such as ligation, competent cell preparation and transformation were followed, as previously described (17). Recombinant vectors were transformed into competent *E.coli* BL21 (DE3). The recombinant clone(s) harboring plasmid DNA with inserts were screened based on their ampicillin resistance. The fidelity

of *E.coli* BL21 (DE3) transformants was verified by colony-PCR using T7 universal primers. Also, recombinant plasmids were confirmed by restriction enzyme digestion and sequencing (Bioneer, South Korea).

Expression of Chimeric OMP25-BLS Construct

In order to express recombinant proteins, the positive pET-32a(+) colonies were cultured on the LB medium containing ampicillin. Protein synthesis was induced with 0.1 mM isopropyl β-D-thiogalactoside (IPTG) in a culture of bacteria with an OD = 0.6. The bacterial culture was incubated for 5 h at 37°C, and then harvested by centrifugation (3000 g, 20 min and 4°C). The pellet from a 300 ml bacterial culture was suspended in lysis buffer (Tris 50 mM, EDTA 5.0 mM, urea 8.0 M, pH = 8.0) and lysate by sonication for 10 min with 20s intervals between pulses. Cell lysate was subjected to centrifugation at 9000 g for 15 min at 4°C to separate the supernatant containing soluble materials from the pellet. Both the supernatant and the pellet were evaluated on SDS-PAGE 10%, and visualized by coomassie-blue staining so as to analyze the expression of recombinant proteins.

Purification of the Recombinant Protein

The expressed protein was purified by chromatography through Ni-agarose from the insoluble phase of lysate using guanidine hydrochloride 6 M to dissolve the pellet, according to the manufacturer's protocol (Thermo Scientific, USA).

The quality and identity of the purified recombinant protein were analyzed by the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method and the western blotting assay, respectively. With regard to the western blotting, the SDS-PAGE gel was electroblotted onto nitrocellulose. The blotted nitrocellulose was then blocked with skim milk for 3h. The membrane was washed three times with phosphate buffered saline with Tween 20 (PBST; PBS with 0.1% (v/v) Tween) and then anti poly-histidine-HRP (Sigma, USA) (1:2000 diluted in BSA 1%) was added to it. After 1 h of incubation at room temperature and washing four times with PBST, diaminobenzidine (DAB), as chromogen, was employed for visualization. Finally, the quantity of the recombinant protein was estimated using Bradford assay. This assay is based on the binding of the dye coomassie blue G-250 to the protein and measures the optical absorbance at 595 nm wavelength. The purified proteins were stored at -20°C for further evaluation of immunogenicity and protective efficacy in mice.

Results

Amplification, Cloning and Nucleotide Acid Sequencing

Figure 1 shows the chimeric OMP25-BLS was amplified using SOE-PCR technique.

In our previous study (9), the OMP25 (642 bp) and the BLS (477 bp) genes were amplified and cloned (Figure 2A) and used as the template for amplifying chimeric OMP25-BLS construct. The accuracy of OMP25-BLS construct with the

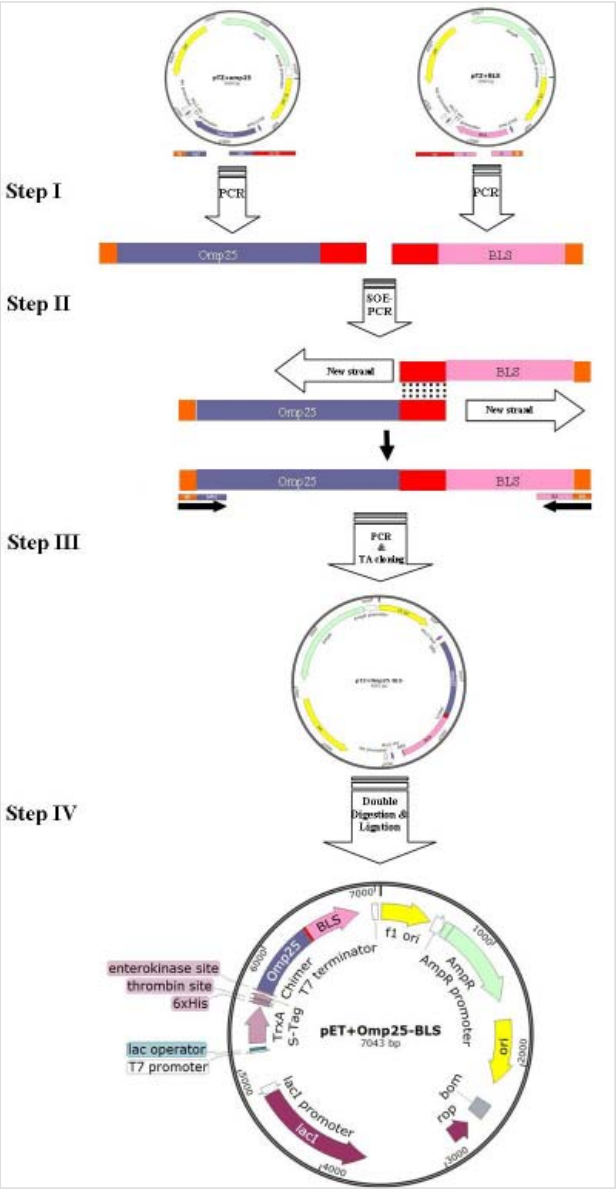

Figure 1: The schematic picture of different steps for designing chimeric OMP25-BLS construct. Step I, Amplification of genes with proper linker primers; Step II, SOE PCR in order to amplify chimeric constructs; Step III, T/A cloning of chimeric PCR products in cloning vector pTZ57R/T; Step IV, Subcloning of OMP25-BLS chimeric fragment in pET-32a(+)

length of 1163 bp visualized on agarose gel electrophoresis (Figure 2B).

Amplicons were successfully ligated into cloning vector pTZ57R/T, OMP25-BLS then was subcloned into pET-32a(+) vector, and transformed into the expression competent cells. After selection of positive colonies by using colony-PCR, the integrity of the recombinant plasmids

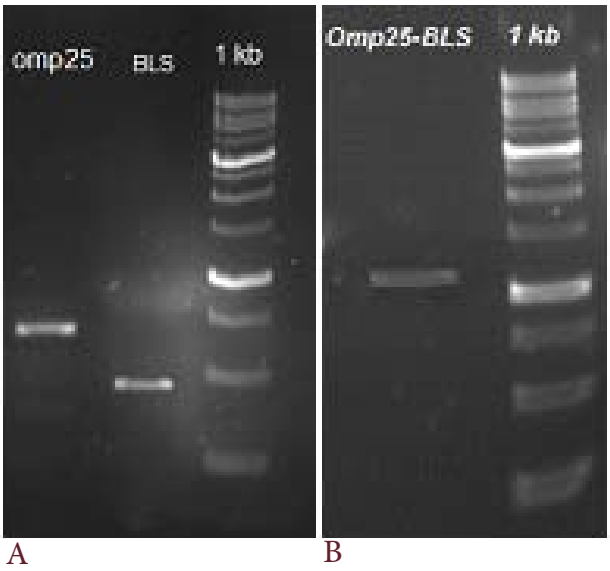

Figure 2: A: Electrophoresis of PCR products of Omp25 and BLS genes on agarose gel 1%; B: PCR products of OMP25-BLS construct on agarose gel 1%

was confirmed by restriction enzyme digestion (*NcoI* and *BamHI*) (Figure 3). And the chimeric construct was sequenced by means of specific primers as well as universal primers.

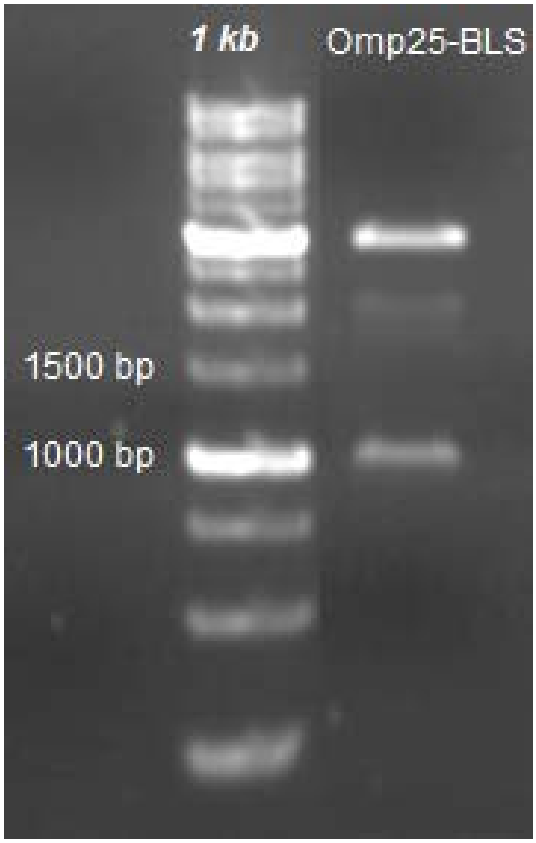

Figure 3: The double digest of construct using NcoI and BamHI restriction enzymes

Expression and Purification of the Recombinant Protein

The expression of the recombinant construct was induced with 0.1, 0.2 and 0.4 mM IPTG. The results of induction with different concentrations of IPTG were the same. Further, recombinant construct was expressed with 0.1 mM IPTG at OD = 0.6 for 5 h at 37°C. After inducing with IPTG, the expected recombinant protein band was detected on SDS-PAGE. The recombinant protein was purified by Ni-NTA affinity chromatography using denaturing method. The purity and identity of the recombinant protein were assessed by both the SDS-PAGE method and the western blotting assay. The lysate supernatant of *E. coli* BL21 (Figure. 4A) and the purified protein (Figure 4B) demonstrated the expected recombinant protein with approximately 59KDa molecular mass on SDS-PAGE (10%). The Western blotting with anti poly-histidine-HRP antibody revealed the specificity of the purified recombinant construct as produced in *E. coli* cells and showed the functional expression in the prokaryotic system (Figure 4C).

Discussion

Recently many efforts have been committed to find out novel immunogens in *Brucella* through different immune approaches (1). The main consideration as the practical steps for introducing new vaccines is the selection and production of novel vaccine candidates (18). Major OMPs of *Brucella* species have been defined as immunogenic and protective antigens (19). OMP25, as one of the major OMP of *Brucella* species, is indulged in virulence (8). It has shown (11) that it could inhibit TNF-α production in human macrophages infected by *B. suis*. TNF-α is an important component of the cytotoxic immune response needed for the

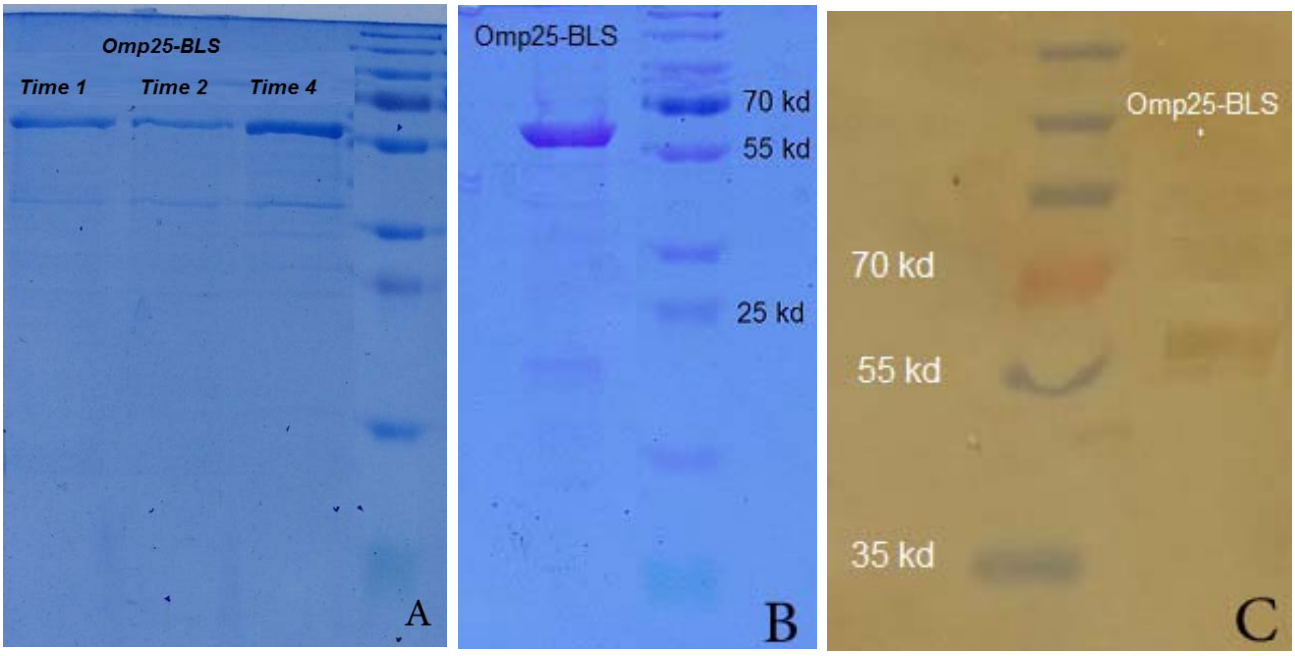

Figure 4: A: SDS-PAGE analysis of the recombinant protein with different sampling time showing the expression after inducing by 0.1 mM IPTG (sampling after 1, 2 and 4 hours); B: The purified protein using Ni-NTA affinity chromatography; C: The Western blotting profile of the recombinant protein (Thermo Scientific Pre-stained Protein Marker with 9 bands)

Brucella clearance from macrophages. TNF-α stimulates not only a cytokine cascade involving IFN-ϒ followed by stimulating natural killer cells for cytotoxicity, but also stimulates the phagocytic activity of macrophages, and induces apoptosis in infected macrophages (20). There are reports demonstrating the DNA vaccines based on OMP25 of *B. melitensis* are protective against the virulent *B. melitensis* challenge in mice (21). Goel and Bhatnagar (20) showed that the intradermal immunization with less amount of recombinant OMP25 generate higher antibody titre in mice than the intraperitoneal immunization even with a high amount of protein. BLS as another *Brucella* antigen has been shown elaborating partial protection against *B. abortus* not only as a DNA vaccine (22), but also as a recombinant protein (23). This antigen additionally having adjuvant properties that seems being an effective activator of bone marrow dendritic cells (24,25). BLS has also been identified to be an antigen delivery system with oral immunity property (12). Also, Velikovsky, et al. (23) showed that BLS can elicit a mixed Th1-Th2 immune response, by which the infection in mice as challenged with *B. abortus* 544 is effectively reduced. Clausse, et al. (26) showed that the recombinant chimeric BLS-OMP31 could be a useful candidate for the development of a subunit vaccine against *B. canis*, since they elicited antigen-specific humoral and cellular responses and conferred protection in the mouse model. Also, Estein, et al. (27) and Diaz, et al. (28) determined that the polymeric subcellular vaccine BLS-OMP31 confers protection against experimental challenge with *B. ovis*.

In the present study, a chimeric OMP25-BLS antigen was designed and successfully produced as a primary step in order to introduce new vaccines to motivate the immune system against *Brucella* infection. Due to the importance of high level production of the recombinant protein in immunological studies, the such constructs are cloned into pET-32a(+) expression vector. Based on pET-32 Xa/LIC vector kit - Novagen protocol, the pET-32a(+) vector is commonly being designed for both cloning and high-level expression recombinant protein sequences fused with the 109aa Trx•Tag™ thioredoxin protein. Cloning enzyme sites, in the pET series, make it possible to produce fusion proteins that have cleavable His•Tag® and S•Tag™ sequences for detection and purification.

The integrity of the synthesized chimeric OMP25-BLS was confirmed by sequencing. The recombinant plasmid construct was then transformed into *E. coli* BL21 (DE3) as the expression host containing T7 RNA polymerase. Different concentrations of IPTG were finally used to induce the expression and the high efficiency of the designed construct was demonstrated by a high level production of the OMP25-BLS.

Conclusion

Brucellosis is a common zoonotic disease that can infect domestic animals and unfortunately there is still no any recombinant vaccine against it. So, in the present study, we could introduce a novel chimer-

ic OMP25-BLS antigen to introduce new subunit vaccines for stimulating the immune system against *Brucella* infection. Also, based on confidence for validity, we have already initiated the evaluation of humoral and cellular immune responses of such constructs against *Brucella melitensis* infection in our laboratory.

Acknowledgement

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Conflict of Interest

The authors declare that they have no conflict of interest

References

1. Golshani M, Rafati S, Jahanian-Najafabadi A, et al. In silico design, cloning and high level expression of L7/L12-TOmp31 fusion protein of Brucella antigens. Res Pharm Sci 2015;10(5):436-45.
2. Pappas G, Papadimitriou P, Christou L, Akritidis N. Future trends in human brucellosis treatment. Expert Opin Investig Drugs 2006;15(10):1141-9.
3. Franco MP, Mulder M, Gilman RH, Smits HL. Human brucellosis. Lancet Infect Dis 2007;7(12):775-86.
4. Corbel MJ, Organization WH. Brucellosis in Humans and Animals: World Health Organization; 2006.
5. Cassataro J, Estein SM, Pasquevich KA, et al. Vaccination with the recombinant Brucella outer membrane protein 31 or a derived 27-amino-acid synthetic peptide elicits a CD4+ T helper 1 response that protects against Brucella melitensis infection. Infect Immun 2005;73(12):8079-88.
6. Seleem MN, Boyle SM, Sriranganathan N. Brucellosis: a re-emerging zoonosis. Vet Microbiol 2010;140(3-4):392-8.
7. Gupta VK, Vohra J, Kumari R, Gururaj K, Vihan VS. Identification of Brucella isolated from goats using PstI sitepolymorphism at Omp2 gene loci. Ind J Animal Sci 2012;82(3):240-43.
8. Edmonds MD, Cloeckaert A, Elzer PH. Brucella species lacking the major outer membrane protein Omp25 are attenuated in mice and protect against Brucella melitensis and Brucella ovis. Vet Microbiol 2002;88(3):205-21.
9. Tahmoorespur M, Sekhavati MH, Yousefi S, Abbassi-Daloii T, Azghandi M, Akbari R. In silico analysis of Omp25 and BLS Brucella melitensis antigens for designing subunit vaccine. Archives of Razi Institute 2016;71(1):35-42.
10. Edmonds MD, Cloeckaert A, Booth NJ, et al. Attenuation of a Brucella abortus mutant lacking a major 25 kDa outer membrane protein in cattle. Am J Vet Res 2001;62(9):1461-66.
11. Jubier-Maurin V, Boigegrain RA, Cloeckaert A, et al. Major outer membrane protein Omp25 of Brucella suis is involved in inhibition of tumor necrosis factor alpha production during infection of human macrophages. Infect Immun 2001;69(8):4823-30.
12. Rosas G, Fragoso G, Ainciart N, et al. Brucella spp. lumazine synthase: a novel adjuvant and antigen delivery system to effectively induce oral immunity. Microbes Infect 2006;8(5):1277-86.
13. Delpino MV, Estein SM, Fossati CA, Baldi PC, Cassataro J. Vaccination with Brucella recombinant DnaK and SurA proteins induces protection against Brucella abortus infection in BALB/c mice. Vaccine 2007;25(37-38):6721-9.
14. Yousefi S, Tahmoorespur M, Sekhavati MH. Cloning, expression and molecular analysis of Iranian Brucella melitensis Omp25 gene for designing a subunit vaccine. Research in Pharmaceutical Sciences 2016;11(5):412-18.
15. Lu P, Feng MG. Bifunctional enhancement of a beta-glucanase-xylanase fusion enzyme by optimization of peptide linkers. Appl Microbiol Biotechnol 2008;79(4):579-87.
16. Sekhavati MH, Tahmoorespur M, Abbassi-Daloii T, et al. Dual Promoter Vector Construction for Simultaneous Gene Expression Using Spliced Overlap Extension by Polymerase Chain Reaction (SOE-PCR) Technique. Iranian Journal of Applied animal Science 2015;5(4):853-58.
17. Sambrook J, Russell DW. Molecular cloning: a laboratory manual. 3rd ed. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press; 2001.
18. Golshani M, Vaeznia N, Sahmani M, Bouzari S. In silico analysis of Brucella abortus Omp2b and in vitro expression of SOmp2b. Clinical and Experimental Vaccine Research 2016;5(1):75-82.
19. Cloeckaert A, Vizcaino N, Paquet JY, Bowden RA, Elzer PH. Major outer membrane proteins of Brucella spp.: past, present and future. Vet Microbiol 2002;90(1-4):229-47.
20. Goel D, Bhatnagar R. Intradermal immunization with outer membrane protein 25 protects Balb/c mice from virulent B. abortus 544. Mol Immunol 2012;51(2):159-68.
21. Commander NJ, Spencer SA, Wren BW, MacMillan AP. The identification of two protective DNA vaccines from a panel of five plasmid constructs encoding Brucella melitensis 16M genes. Vaccine 2007;25(1):43-54.
22. Velikovsky CA, Cassataro J, Giambartolomei GH, et al. A DNA vaccine encoding lumazine synthase from Brucella abortus induces protective immunity in BALB/c mice. Infect Immun 2002;70(5):2507-11.
23. Velikovsky CA, Goldbaum FA, Cassataro J, et al. Brucella Lumazine Synthase Elicits a Mixed Th1-Th2 Immune Response and Reduces Infection in Mice Challenged with Brucella abortus 544 Independently of the Adjuvant Formulation Used. Infect Immun 2003;71(10):5750-55.
24. Berguer PM, Mundinano J, Piazzon I, Goldbaum FA. A polymeric bacterial protein activates dendritic cells via TLR4. J Immunol 2006;176(4):2366-72
25. Cassataro J, Pasquevich KA, Estein SM, et al. A DNA vaccine coding for the chimera BLSOmp31 induced a better degree of protection against B. ovis and a similar degree of protection against B. melitensis than Rev.1 vaccination. Vaccine 2007;25(32):5958-67
26. Clausse M, Diaz AG, Ghersi G, et al. The vaccine candidate BLSOmp31 protects mice against Brucella canis infection. Vaccine 2013;31(51):6129-35
27. Estein SM, Cassataro J, Vizcaino N, Zygmunt MS, Cloeckaert A, Bowden RA. The recombinant Omp31 from Brucella melitensis alone or associated with rough lipopolysaccharide induces protection against Brucella ovis infection in BALB/c mice. Microbes Infect 2003;5(2):85-93
28. Diaz AG, Clausse M, Paolicchi FA, et al. Immune response and serum bactericidal activity against Brucella ovis elicited using a short immunization schedule with the polymeric antigen BLSOmp31 in rams. Vet Immunol Immunopathol 2013;154(1-2):36-41

The better view of picture 1 in detail: